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Ascorbic acid accumulation in plated human neutrophils

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Ascorbic acid uptake was investigated in isolated, plated human neutrophils using high-performance liquid chromatography with coulometric electrochemical detection. Freshly isolated neutrophils contained 1.3 mM ascorbic acid and accumulated significantly greater amounts when physiologic concentrations of the vitamin were present in the extracellular buffer. In several different buffers uptake was dependent on the presence of calcium and magnesium. Under these conditions, scintillation spectrometry of [^{14}C]ascorbic acid in conjunction with high-performance liquid chromatography was suited for measuring ascorbic acid transport.

Ascorbic acid; Vitamin C; Transport; Neutrophil

1. INTRODUCTION

Ascorbic acid is found in the micromolar concentration range in human neutrophils [1-3]. However, the precise biochemical role of ascorbic acid in neutrophils is not well understood. Ascorbic acid has been suggested to either promote oxidative destruction of microorganisms [4,5] or to preserve neutrophil integrity and/or protect host tissues by inactivating free radicals and oxidants [6,7]. To delineate the role of ascorbic acid in neutrophils it is necessary to study neutrophil function in relation to the concentration of intracellular ascorbic acid. Manipulation of neutrophilic intracellular ascorbic acid content requires an understanding of how ascorbic acid is transported into neutrophils under physiologic conditions. This paper describes some of the conditions necessary for ascorbic acid uptake and accumulation in isolated human neutrophils.

2. MATERIALS AND METHODS

Neutrophils were isolated from heparinized whole blood collected from healthy volunteers as described previously [3] and suspended in Hanks' balanced salt solution without calcium, magnesium, or Phenol red (mHBSS) (pH 7.4) at a final concentration of 1×10^7/ml. Neutrophils were routinely >95% pure and >95% viable as revealed by Wright's staining and exclusion of Trypan blue, respectively.

Transport and accumulation of ascorbic acid in neutrophils were studied using freshly isolated neutrophils plated on 24-well (16 mm diameter) culture plates (Costar, Cambridge, MA) at $0.25\text{-}2.5 \times 10^6$ cells/well in 100 μl mHBSS. To each well was added 900 μl of either a bicarbonate-free suspension buffer [8] modified to contain 10 mM Hepes, Hanks' balanced salt solution without Phenol red, Dulbecco's phosphate-buffered saline, or Krebs-Ringer bicarbonate buffer. All buffers contained 5 mM glucose (pH 7.4). The culture plates were incubated in a humidified 5% CO_2 atmosphere at 37°C and the cells allowed to adhere to the well bottoms for 40 min. Before the start of each experiment the nonadherent cells and buffer were removed and the adherent cells washed once with 1 ml of the appropriate buffer. Fresh buffer of the appropriate type with or without calcium and magnesium, containing 5 mM glucose, and with 50 μM ascorbic acid or 300 μM [^{14}]ascorbic acid (DuPont-New England Nuclear) (pH 7.4) was then added to each well to give a final volume of 1 ml. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. At the various times indicated in the text the suspension buffer was removed, the cells washed and extracted, and then stored as previously described until analyzed for ascorbic acid and protein content [3].

High-performance liquid chromatography was used to measure total intracellular ascorbic acid, as previously described [3]. Where applicable, [^{14}C]ascorbic acid elution profiles of the samples were obtained by this method. Purity of the stock [^{14}C]ascorbic acid solution dissolved in 30% methanol, 1 mM EDTA was also determined by high-performance liquid chromatography both before and after addition of ascorbic acid oxidase [9]. For both samples and stock isotope solution the column eluant was collected in 6 s fractions over a 3 min time interval starting at the time of injection using an automate fraction collector (Pharmacia LKB Biotechnology). The collected fractions were counted in a liquid scintillation counter (model LS-5801, Beckman Instruments).

Intracellular ascorbic acid concentration was determined by conversion of neutrophil protein to intracellular volume, as previously described [3]. Experimental points represent the mean ± S.D. of 3 samples. Error bars have been omitted where the SD was less than the size of the symbol.

3. RESULTS

We found that isolated human neutrophils contained approximately 1.3 mM intracellular ascorbic acid and had the capacity to accumulate significantly greater amounts when ascorbic acid was present in the extracellular buffer (fig.1). Neutrophils incubated with 50 μM ascorbic acid accumulated ascorbic acid in a linear

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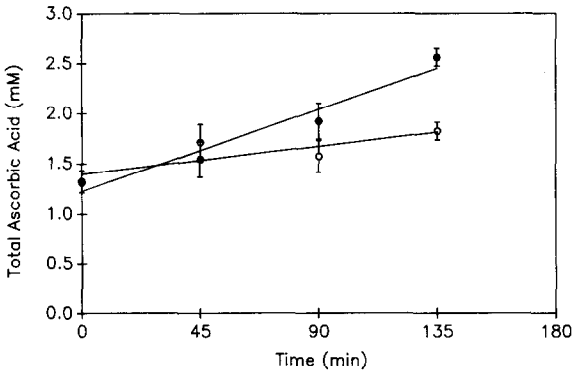

Fig.1. Ascorbic acid accumulation in neutrophils as a function of time. Adherent neutrophils were incubated in bicarbonate-free buffer containing 50 μM ascorbic acid with (●) or without (○) 1.5 mM Ca^{2+} and 1.3 mM Mg^{2+} (pH 7.4) for the times indicated.

fashion for more than 2 h. Uptake was dependent on the presence of calcium and magnesium in the extracellular buffer (fig.1). In the presence of these cations the intracellular ascorbic acid doubled from 1.3 to 2.6 mM, while in their absence intracellular ascorbic acid increased from 1.3 to 1.8 mM. These data indicate that calcium and magnesium increased ascorbic acid transport.

We further investigated the effect of calcium and magnesium by studying ascorbic acid accumulation using different buffers. We chose 4 buffers commonly used with neutrophils. As seen in table 1, neutrophils accumulated ascorbic acid in a remarkably similar fashion in bicarbonate-free buffer, Hanks' balanced salt solution, and Dulbecco's phosphate-buffered saline. In the presence of calcium and magnesium, ascorbic acid accumulation was increased for each buffer. Neutrophils accumulated less ascorbic acid in Krebs-Ringer bicarbonate buffer in the presence or absence of calcium and magnesium for unclear reasons. This was probably not due to a difference in sodium, calcium, or magnesium content, since Krebs-Ringer bicarbonate buffer has an equal amount of sodium compared to the other buffers and has slightly more calcium and magnesium than the other 3 buffers. These data demonstrate a requirement for divalent cations for maximal ascorbic acid transport, independent of buffer. In addition, except for Krebs-Ringer bicarbonate buffer, these buffers appear to be equally suited to study ascorbic acid transport in these cells.

Since these cations can only be used in plated neutrophils, due to cation-induced aggregation of free-floating neutrophils, plated cells were chosen for experiments. Thus, it was necessary to determine the relationship between the number of neutrophils plated versus the protein and ascorbic acid content per well. As seen in fig.2, there is a linear relationship between the number of cells per well and the protein and ascorbic acid content between 0.25×10^6 and 2.5×10^6 cells/well. At higher cell concentrations, the measured ascorbic acid and protein contents were lower than expected, indicating that the wells cannot accomodate more than 2.5×10^6 neutrophils. Based on these data, we used a plated concentration of 1.0×10^6 neutrophils/well.

We also observed that approximately 50% of the neutrophils originally added to each well were lost during the first wash procedure, as determined by comparing the protein and ascorbic acid contents of plated cells with free-floating cells. The percentage of adherent cells was constant over the range $0.5–2.5 \times 10^6$ cells/well. However, there was no subsequent loss of adherent cells upon additional washings (data not shown). Therefore, analytical data were based on a protein basis per well rather than the number of cells originally added per well, to account for nonadherent cell loss. After accounting for nonadherent cells, we found that plated and free-floating neutrophils had the same concentration of ascorbic acid (data not shown).

Since ascorbic acid is transported by human neutrophils, it seemed possible to use [^{14}C]ascorbic acid to measure transport under these conditions. However, before radiolabelled material could be used, it was essential to determine if intracellular radiolabelled

Table 1

Effect of buffer type on the accumulation of ascorbic acid (mM) in isolated human neutrophils

Cations	Incubation buffer			
	BFB	HBSS	DPBS	KRB
− Ca^{2+}, Mg^{2+}	1.82 ± 0.10	2.03 ± 0.10	1.97 ± 0.11	1.47 ± 0.25
+ Ca^{2+}, Mg^{2+}	2.56 ± 0.09	2.46 ± 0.11	2.53 ± 0.40	1.67 ± 0.15

1×10^6 plated neutrophils were incubated for 180 min in 1.0 ml of bicarbonate-free buffer (BFB), Hanks' balanced salt solution without Phenol red (HBSS), Dulbecco's phosphate-buffered saline (DPBS), or Krebs-Ringer bicarbonate buffer (KRB) with or without calcium and magnesium. All buffers contained 50 μM ascorbic acid and 5 mM glucose (pH 7.4). Initial internal ascorbic acid concentration was 1.30 ± 0.11 mM under all conditions. Ascorbic acid analysis was performed as described in section 2. Each internal ascorbic acid concentration is the mean ± SD of 3 values

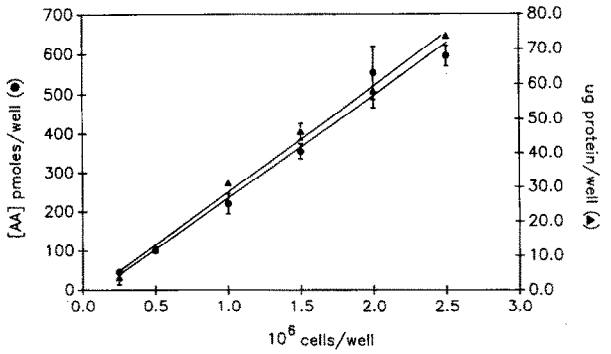

Fig.2. Protein (▲) and ascorbic acid (●) content of neutrophils as a function of number of cells plated. Neutrophils were plated in 1.0 ml of bicarbonate-free buffer containing 1.5 mM Ca^{2+} and 1.3 mM Mg^{2+} (pH 7.4) for 60 min. Number of cells added to each well are indicated in the figure.

material was truly ascorbic acid, due to the inherent instability of the vitamin. We therefore determined the purity of radiolabelled material before and after transport into neutrophils. As shown in fig.3A, a [^{14}C]ascorbic acid standard eluted as a large peak between fractions 18 and 24, with a small earlier peak in fractions 14–18. Approximately 95% of the radioactivity corresponded to the ascorbic acid peak by HPLC.

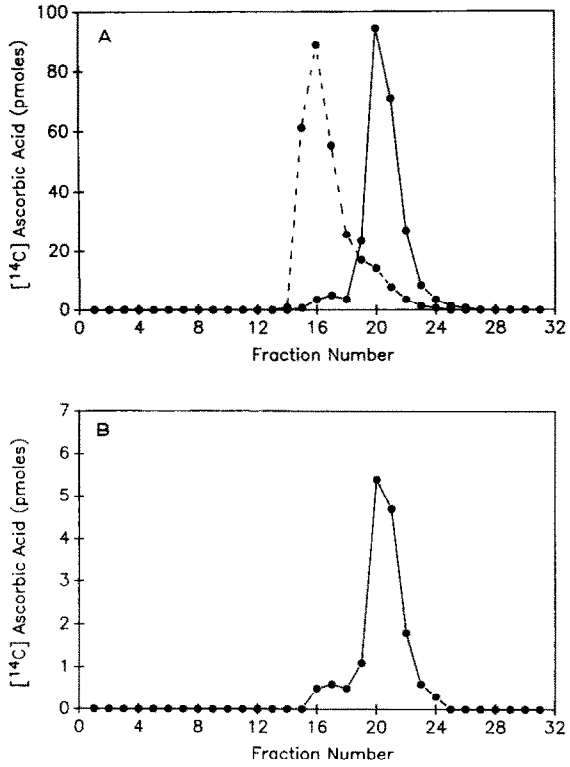

Fig.3. Elution profiles of [^{14}C]ascorbic acid obtained by high-performance liquid chromatography. (A) [^{14}C]Ascorbic acid stock solution containing 600 pmol ascorbic acid per 10 μl injection volume before (——) and after (– – –) treatment with ascorbic acid oxidase. (B) Neutrophil cell extract containing 47 pmol ascorbic acid per 50 μl injection volume.

The small peak in fig.3A was most likely dehydroascorbic acid, since treatment of the radiolabelled ascorbic acid with ascorbic acid oxidase, to produce dehydroascorbic acid, shifted the ascorbic acid peak to fractions 14–18. Fig.3B shows the elution profile of the cell extract obtained from neutrophils cultured in bicarbonate-free buffer containing 300 μM [^{14}C]ascorbic acid (pH 7.4) for 90 min. A large peak between fractions 18 and 24, and a small peak between fractions 16 and 18 were obtained, in agreement with the elution profile of the [^{14}C]ascorbic acid stock solution. These data indicate that intracellular radiolabelled material is ascorbic acid, under the incubation conditions used here.

4. DISCUSSION

These data indicate that human neutrophils accumulate ascorbic acid and identify conditions suitable for measurement of ascorbic acid transport and accumulation. Maximal ascorbic acid accumulation occurred in the presence of calcium and magnesium. Although other cells also require these cations for ascorbic acid uptake, the mechanism is unknown [10,11]. In neutrophils it is possible that calcium and magnesium are part of a co-transport system with ascorbic acid, as has been suggested for sodium and ascorbic acid in other cells [10,12]. However, since neutrophils accumulate some ascorbic acid even in the absence of calcium and magnesium other factors may be involved, such as the existence of more than one transporter with different ion requirements. Furthermore, since divalent cations promote neutrophil adherence, granule exocytosis, and translocation of intracellular membranes at the cell surface [13–15], it is possible that these adherence-related events are involved in the divalent cation-dependent enhancement of ascorbic acid transport.

The results also indicated that radiolabeled ascorbic acid remained as ascorbic acid after transport into human neutrophils. These data imply that the radiolabelled ascorbic acid can be used for transport studies in human neutrophils. However, we emphasize that quantitation of radiolabel alone should not be used to determine ascorbic acid transport. Depending upon intracellular and extracellular conditions, [^{14}C]ascorbic acid may be oxidized, which would go undetected without a corresponding direct mass measurement. In addition, radiolabel uptake provides no information about endogenous ascorbic acid levels in neutrophils or about total intracellular content. Quantitation of radiolabel uptake in conjunction with mass measurement provides the most accurate information about ascorbic acid uptake.

Since calcium and magnesium were used for ascorbic acid accumulation, characterization of the adherent neutrophil population was essential for accurate quantitation of intracellular ascorbic acid. Thus, transport

studies were optimized regarding number of neutrophils plated per well. These experiments were necessary, since the data indicated that excessive cell loss occurred when neutrophils were plated above a density of 2.5×10^6 cells/well. The experiments also showed that ascorbic acid accumulation should be expressed as a function of adherent cells remaining and not as a function of cells originally plated. These data provide a foundation for studying ascorbic acid transport in resting and activated human neutrophils.

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